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SEROTONINERGIC REGULATION
OF
SECRETORY ACTIVITY
OF THE SUBCOMMISSURAL ORGAN
IN SOME RODENTS

BY

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- II. Madsen, J.K. & Møllgård, K.: The tight epithelium of the Mongolian gerbil subcommissural organ as revealed by freeze fracturing. *J. Neurocytol.* 8 (1979) in press.
- III. Kimble, J.E. & Møllgård, K.: Evidence for basal secretion in the subcommissural organ of the adult rabbit. *Z.Zellforsch.* 142, 223-239 (1973).
- IV. Kimble, J.E. & Møllgård, K.: Subcommissural organ-associated neurons in fetal and neonatal rabbit. *Cell Tiss.Res.* 159, 195-204 (1975).
- V. Wiklund, L., Lundberg, J.J. & Møllgård, K.: Species differences in serotonergic innervation and secretory activity of rat, gerbil, mouse and rabbit subcommissural organ. *Acta physiol. scand. Suppl.* 452, 27-30 (1977).
- VI. Møllgård, K., Lundberg, J.J., Wiklund, L., Lachenmeyer, L. & Baumgarten, H.G.: Morphologic consequences of serotonin neurotoxin administration: Neuron-target cell interaction in the rat subcommissural organ. *Ann.N.Y.Acad.Sci.* 305, 262-288 (1978).
- VII. Møllgård, K. & Wiklund, L.: Serotonergic synapses on ependymal and hypendymal cells of the rat subcommissural organ. *J.Neurocytol.* 8 (1979) in press.
- VIII. Wiklund, L. & Møllgård, K.: Neurotoxic destruction of the serotonergic innervation of the rat subcommissural organ is followed by reinnervation through collateral sprouting of non-monoaminergic neurons. *J.Neurocytol.* 8 (1979) in press.

These papers will be referred to by their Roman numerals.

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INTRODUCTION

The subcommissural organ (SCO) is a specialized area of the ependyma attached to the ventral surface of the posterior commissure. It forms the lining of the roof of the cerebral aqueduct at its junction with the third ventricle and releases secretory material of glycoprotein character apically into the ventricular fluid, where it in most species condenses to form the Reissner's fiber (RF). Basal secretion of unknown hormone-like material into the blood vessels of the SCO is postulated in some species.

The history of the SCO began at the turn of the century with a number of careful histological studies, although the term "subcommissural organ" was introduced first in 1910 by Dendy & Nicholls. The histology and comparative anatomy of the SCO was thus quite well known in 1925 when Krabbe brought that line of research to a close by discovering and describing a basal layer in the organ which he named the hypendyma.

A detailed histochemical and functional analysis of the SCO was initiated by the findings of Stutinsky (1950), who demonstrated the presence of so-called neurosecretory (Gomori-positive) material in the organ. The stainable material is considered to be a neutral mucopolysaccharide-protein complex (Bargmann & Schiebeler, 1952; Wislocki & Leduc, 1952b) with a high cystine content (Oksche, 1962; Naumann, 1968). Some extensive enzyme-histochemical investigations have led to the conclusion that the SCO is characterized by a high glycogenolytic and glycolytic, but low oxidative capacity (Köhl, 1975, for a recent review see Ziegels, 1976).

The functional significance of the SCO-RF complex is still a matter of speculation, although an impressive number of different hypotheses has been proposed and tested during the last century. The search goes on and new proposals from the last decade include (1) that the Reissner's fiber is involved in morphogenetic processes by promoting the normal straight growth of the spinal cord and vertebral column (Rühle, 1971), (2) that it plays a role in stimulating the liquor contacting

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neurons in the central canal (Vigh et al., 1970), (3) that it selectively may bind some biogenic amines and their metabolites within the cerebrospinal fluid (Hess & Sterba, 1973) and thus contributes to the regulation of its composition (Diederer, 1975, also cf. Olsson, 1958).

One of the most persistent theories postulates that the mammalian SCO is involved in the control of thirst, water balance and/or osmoregulation (cf. Palkovits, 1965; Diederer, 1975). This was initially proposed by Gilbert (1956), who reported that destruction of the organ in rats resulted in permanent adipsia, that injection of an SCO extract into normal rats caused a temporary hypodipsia (Gilbert, 1957), and that the stainability of the SCO varied with changes in hydrated-dehydrated states of the rat (Gilbert, 1958). Many investigators in the field have confirmed and extended these findings, but other studies have failed to detect any antidiuretic principle in the SCO and any change in its activity in relation to osmotic variations (for references, see reviews by Palkovits, 1965; Sterba, 1969; Diederer, 1975). Most of these experimental studies have unfortunately been performed in adult rats in which the SCO exhibits a very low secretory activity.

The present thesis work has established five factors that go beyond species differences and should be considered when evaluating the secretory activity of the SCO:

- 1) The SCO-area specification. Variations exist between rostral and caudal parts of a particular mammalian SCO, and a number of regional differences have been demonstrated in earlier publications (e.g. Møllgård, 1972; Møllgård et al., 1973; Møllgård, 1974).
- 2) The barrier properties and secretory polarity of the SCO in question (papers I, II, III).
- 3) The presence or lack of SCO-associated neurons (papers IV).
- 4) The presence or lack of innervation (paper V, unpublished data).
- 5) The changes in morphological features during development (paper V, unpublished data).

Previous fluorescence histochemical studies have revealed the presence of serotonin-containing terminals in association with the SCO in the rat (for references, see p.17). In the present series of investigations it was found that these nerves make direct synaptic connections with the secretory SCO cells (paper VII). These studies also propose that the SCO of the rat might serve as an ideal model for serotonergic synaptic regulation of metabolic and secretory activity (papers VI, VII and VIII).

MATERIAL AND METHODS

ANIMALS

More than 300 fetal, neonatal, juvenile and adult rabbits, rats, Mongolian gerbils and mice of both sexes were investigated. All experimental animals were kept at room temperature and natural light-dark rhythm. Most of the animals were killed between 10 and 11 a.m. Some were killed at 11 p.m. and showed no obvious difference in SCO secretory activity. There were also no definite seasonal changes and no difference between the secretory activity of the SCO of males and females.

ELECTRON MICROSCOPY (RABBITS, RATS AND GERBILS)

Thin section electron microscopy. Under Brietal[®] (Lilly) anesthesia (40 mg/kg, i.p.) gerbils and rats were fixed by perfusing a solution containing 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at a constant pressure of 140 mm Hg through the left ventricle. Rabbits were anesthetized with Nembutal[®] (30 mg/kg), and artificially respired with a small animal respirator (Harvard apparatus). A catheter was placed in the abdominal aorta, and the brains were fixed by perfusing a solution containing 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) through the aortic catheter at a pressure of 130-140 mm Hg during the first min., 80 mm Hg during the following two min. and 30-40 mm Hg during the rest of the perfusion. These measurements were recorded by a second catheter situated at the tip of the infusion catheter. Prior to fixation the intra-aortic pressure was about 90 mm Hg. After 15-20 min. of perfusion fixation, the brains of all experimental animals were removed and placed in fresh fixative for 6 hr. Tissue blocks

that contained the SCO were isolated and post-fixed for 2 hr in 2% OsO_4 in 0.1 M cacodylate buffer. Specimens were block stained with uranyl acetate for 1 hr, quickly dehydrated in increasing concentrations of ethanol, and embedded in epon. The blocks were carefully oriented during embedding so that survey sections would be strictly in the sagittal or frontal plane. One micron thick sections stained with toluidine blue were examined in the light microscope. Silver-to-gray thin sections were cut from selected areas and, after poststaining with uranyl acetate and lead citrate, viewed in a Hitachi HS8 microscope.

Ventricular perfusion of ruthenium red. In some rabbits the ventricular system was perfused from one lateral ventricle to the cisterna magna. The ventriculo-cisternal perfusion was performed according to the method described by Oldendorff and Davson (1967). Intracranial pressure and arterial blood pressure were monitored continuously in order to assure that these parameters remained normal during the ventriculo-cisternal perfusion. In two of these rabbits the ventricular system was first perfused with mock (artificial) cerebrospinal fluid (CSF) at a rate of 0.35 cc/min for 1 hour. Thereafter, the brains were fixed by ventriculo-cisternal perfusion with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). In other rabbits the ventricular system was perfused for 1 hour with mock CSF containing ruthenium red (1000 p.p.m.), and the brains were thereafter fixed by ventriculo-cisternal perfusion with a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer and ruthenium red (1000 p.p.m.) as during the previous perfusion with mock CSF.

The osmolarities of the above-mentioned perfusates were as follows: mock CSF, 294 m.osm.; mock CSF + ruthenium red (1000 p.p.m.), 303 m.osm.; glutaraldehyde + buffer, 526 m.osm.; glutaraldehyde + buffer + ruthenium red (1000 p.p.m.), 505 m.osm. Following fixation by ventriculo-cisternal perfusion, the brains were removed and tissue blocks containing the SCO were processed for electron microscopy as described above.

Freeze-fracture electron microscopy. Small blocks of SCO of rabbits, rats and Mongolian gerbils were prepared after 6 hr of fixation described above. Following 2 hr of infiltration with 25% buffered glycerol at room temperature the specimens were mounted and oriented on gold disks so that a transverse cleavage perpendicular to the ventricular surface could be achieved. The tissue was then frozen in the liquid phase of partially solidified Freon 22 and stored in liquid nitrogen. Freeze-fracturing followed by carbon-platinum shadowing was performed with a Balzer's apparatus (BAF 301) equipped with an electron beam gun. Specimens were fractured at a stage temperature of -115°C and replicated with platinum-carbon without etching. The thickness of the platinum-carbon and carbon coats (approximately 2 nm and 20 nm) was controlled by a thin film monitor. Replicas were cleaned in chromic acid and chrome-sulphuric acid.

HISTOCHEMISTRY (RABBITS, RATS, GERBILS AND MICE)

Fixation and preparation. Ice-cold fixation was performed with either formol-calcium (pH 5.3) or Bouin's fixative (Pearse, 1968). Fixation time was 24 to 72 hr in the case of Bouin fixation. Formol-calcium fixation was normally 24 hr. Serial paraffin sections of 3-5 micron were cut in sagittal or frontal planes. For fluorescence histochemistry - see below.

Methods.

I. Carbohydrates and mucosubstances. 1. Alcian blue (0.05%) I.C.I. - critical electrolyte concentration (Scott & Dorling, 1965). 2. Periodic acid-Schiff. The PAS-method was used with de Tomasi's reagent (Pearse, 1968). As a control some sections were digested by Maltase (Merck) before staining and other sections were stained omitting the periodic acid oxidation. 3. Metachromatic staining with 0.1% toluidine blue 0 (Merck) in 30% ethanol (Kramer & Windrum, 1955). Staining time 10-30 sec.

II. Reactions for "Neurosecretory Substance".

4. Bargmann's chrom hematoxylin Method (Pearse, 1968). 5. Bock's chromalum gallocyanin Method (Bock, 1966). 6. The reaction for cystine (PFA-AB) (Pearse, 1968) was compared with above-mentioned reactions for "neurosecretory substance". 7. Sections digested by ribonuclease (Fluka) (1 mg/ml dist. water, pH 5.6) for 1-4 hr were used as a control in each of the above-mentioned staining methods.

III. Fluorescence Histochemistry. All animals used for fluorescence histochemical detection of monoamines were pharmacologically pretreated according to Aghajanian et al., (1973) to increase intraneuronal serotonin levels. Injection of chloral hydrate (300 or 400 mg/kg, i.p.) was followed 10 min later by 300 mg/kg of nialamide (i.p.); 5 min after the second injection, 100 mg/kg of L-tryptophan (i.p.) were administered.

One to 3 hr after the last injection, the experimental animals were killed by decapitation under light ether anesthesia. The brains were rapidly dissected, and blocks that contained the SCO were quickly frozen in liquid propane, cooled by liquid

nitrogen, and subsequently freeze-dried (for technical details, see Björklund et al., 1972). To achieve a high fluorescence yield of serotonin, a two-step formaldehyde gas treatment was used (Fuxe & Jonsson, 1967).

AUTORADIOGRAPHY (RATS)

The rats used for autoradiographic localization of (^{14}C)-5,6 DHT were fixed by vascular perfusion with a cold saline solution that contained 4% acid-free formalin and were embedded in paraffin. Serial 5 μm sections were mounted on glass slides, deparaffinized, and coated with undiluted Ilford L 4 emulsion (dipping technique). Exposure of the emulsion-coated sections was performed at 8°C in darkness for 6 weeks. The silver grains were developed with Kodak D 19 b and fixed with sodium thiosulfate. Subsequently, all sections were counterstained with gallocyanine chromalum (according to Einarson) for 24 hr and mounted in Caedax[®].

ADMINISTRATION OF NEUROTOXIC DRUGS (RATS)

Under barbiturate anesthesia (Brietal[®], 40 mg/kg) rats were injected stereotaxically in the lateral ventricle with one of the following drugs: 50 or 75 μg of 5,6 DHT (Regis), 50 μg of (^{14}C)-5,6-DHT (3 or 14 mCi/mmol), 150 μg of 5,7-dihydroxytryptamine (5,7-DHT, Regis), or 50 μg of 6-hydroxytryptamine (6-HT) (all calculated as free base). All drugs were injected in a volume of 20 μl of saline (which contained L-ascorbic acid, 0.2 mg/ml, as an antioxidant). Normal, untreated rats and rats injected in the lateral ventricle with 20 μl of saline were used as controls. At various time intervals after injection, the rats were processed for either fluorescence microscopy, autoradiography, electron microscopy, or histochemistry.

RESULTS AND COMMENTS

A. Barrier properties and secretory polarity.

The SCO is included in the group of specialized areas of ependyma, which are collectively called circumventricular organs. Although it is often stated that the SCO like other circumventricular organs has no blood-brain barrier (see e.g. Löfgren, 1965 and review by Koella and Sutin, 1967) this does not appear to be the case. Thus Wislocki and Leduc (1952 a) did not include the subcommissural organ in their list of areas outside the blood-brain barrier after an extensive study using trypan blue and silver nitrate. Furthermore, intravenously injected horseradish peroxidase (HRP) did not penetrate into the organ in rabbits (Weindl and Joynt, 1973), rats (von Bomhardt et al., 1974) and mice (Broadwell and Brightman, 1976) indicating that the capillaries of the SCO do not differ from typical brain capillaries with respect to the effectiveness of the blood-brain barrier.

1. The CSF-SCO interface (papers I, II).

In contrast to the usual ependymal lining of the ventricular system the ependyma overlying the circumventricular organs is generally held to be more or less impermeable to CSF-borne dyes and proteins due to the presence of tight junctions between adjacent ependymal cells (Brightman et al., 1975). The tight junctions in areas with fenestrated leaky capillaries presumably serve to maintain the insulation of the bulk of the CNS from unrestricted access of material from the blood, but as stated above the capillaries of the SCO are not fenestrated and exhibit a normal blood-brain barrier.

The CSF-SCO interface was studied in adult rabbits after ventriculo-cisternal perfusion of ruthenium red in a solution of mock CSF for one hour before fixation (paper I). The ruthenium red was found to penetrate both zonulae adherentes and gap junctions between normal ependymal cells, and thereby to reach subependymal layers. Both in the median eminence, where the presence of tight junctions was established previous-

ly by the use of HRP (Reese and Brightman, 1968) and in the subcommissural organ the passage of ruthenium red was stopped at the first tight junction, and gap junctions were outlined only if there was not a preceeding tight junction more apically.

Electron dense tracers like ruthenium red, lanthanum and horseradish peroxidase have proven very useful in distinguishing between various types of cell junctions, but the freeze fracture technique provides the unambiguous criteria for their identification.

The SCO of rabbits and rats was examined by freeze fracture electron microscopy, and the results of the previous study (paper I) were confirmed and extended. Special attention was paid to the SCO of the Mongolian gerbil, a species which has the ability to conserve water under conditions of severe water deprivation (paper II). We worked mainly with the SCO of neonatal gerbils as they demonstrate a very high secretory activity in contrast to adult gerbils (paper V). At all ages investigated the ependymal layer of the SCO exhibited complex and well-formed apical tight junctions. The tight junction network is more extensive neonatally than in adult, and in general, more conspicuous in gerbils than in other animal species. The consistent finding of such very complex tight junctions at the interface between the CSF and the SCO suggests that the SCO ependyma at least in neonatal Mongolian gerbils might function as a tight epithelium with an ability to maintain steep solute gradients and high potential differences. The sealing off of the SCO towards the CSF (the CSF-SCO barrier), and the effective blood-brain barrier in the organ might well be of functional significance and thus contribute to further explorations of the obscure histophysiology of the SCO.

Another implication of the tight CSF-SCO barrier is that secretory material released from SCO-hypendymal cells, which are detached from the ventricular surface, would not be expected to penetrate from the extracellular space of the

SCO out into the CSF. A secretory product from the basal SCO is more likely to be destined for the perivascular space which is in direct communication with the extracellular space of the organ. Thus, the present results support the idea of secretion towards the blood vessels - the so-called basal secretion (see paper III).

2. The SCO as a secretory organ (papers III, VI).

Ultrastructural and histochemical investigations have demonstrated that, in a wide range of species, the SCO has a secretory function (for thorough reviews of the literature see Olsson (1958), Palkovits (1965), Sterba (1969) and Herrlinger (1970). Some basic questions concerning the synthetic machinery, the chemical nature of the secretory material, the packing in various types of granules and the polarity of the secretion (apical and /or basal) are, however, subjects of much dispute. Part of this controversy is clearly a result of marked species differences.

At the ultrastructural level the secretory product of the SCO cells is usually described as consisting of two populations of granules - light secretory sacs ("pale granules") and dense granules (e.g. Papacharalampous et al., 1968; Rodríguez, 1970; Murakami et al., 1972). There is general agreement among most authors that the pale granules are derived from the ER, though their final fate and the role played by the Golgi complex is a matter of controversy (cf. paper III). Although the secretory nature of the flocculent material has been questioned (Smith, 1970), many findings support the idea that the light secretory sacs release their contents apically. Basal secretion into the blood vessels of the mammalian SCO has been postulated in dog (Oksche, 1969) and in rabbit (paper III).

The second population of granules, the dense granules, have also been the subject of much dispute. Some authors (Vigh et al., 1967; Herrlinger, 1970) consider the electron dense granules to be the EM correlate of the Gomori-positive

"neurosecretory substance" seen at the light microscopic level, whereas Smith (1970) has inferred that the so-called secretory granules are most likely lysosomes. A histochemical study at the EM level has shown that only few of the dense granules react positively for acid phosphatase, and therefore, that most of them are not of lysosomal nature (Murakami et al., 1972). Not only the nature, but also the origin and fate of the dense granules are a matter of controversy.

At the light microscopic level Stutinsky (1950) first demonstrated Gomori-positive material in the SCO. Since then the organ from various species has been extensively studied with methods considered selective for neurosecretory material (for critical review and references, cf. Vigh et al., 1967; Oksche, 1969; Møllgård et al., 1973).

Some releasing factors (somatostatin, luteinizing hormone-releasing factor and thyrotropin-releasing factor) and a hormone (arginin vasotocin) have recently been detected in the SCO with immunoperoxidase techniques and by radio-immunoassay (for references cf. paper VI). The significance of these findings is obscure at present.

3. Basal secretion (paper III).

While it is generally approved that the mammalian SCO releases an apical secretory product into the ventricular lumen, the concept of the so-called basal secretion does not seem to be widely accepted. In a recent extensive review based on observations on the SCO of rats, mice, guinea pigs and hamsters, Köhl (1975) questioned some earlier observations that suggested the possibility of basal secretion into hypendymal capillaries of the SCO of human fetuses (Møllgård, 1972) and of adult rabbits (paper III). These and similar objections to the assumptions of a basal secretion are, however, primarily based on findings obtained from species in which the anatomical basis for such a secretory mechanism is not apparent (cf. Oksche, 1969).

The hypendymal region of the SCO of adult rabbit was studied electronmicroscopically with particular emphasis on the area in the immediate vicinity of the capillaries (paper III). The morphology of hypendymal cells and basal processes of ependymal cells showed several features suggesting an involvement in basal secretory mechanisms: (1) a high concentration of heterogeneous granules and vesicles bordering a widened perivascular space; (2) an accumulation of mitochondria; (3) a possible mode of secretion for the membrane-bound flocculent material, involving binding of the sacs by small particles to the lateral membrane and subsequent release into a system of extracellular spaces; (4) widened perivascular spaces which extend out in sacs and channels in between the basal processes and (5) high pinocytotic activity in the hypendymal capillary wall. Nerve cells found in close association with the capillary region could provide a control mechanism for secretion (see below).

B. Neurons and terminals in the hypendyma

The consistent findings of neurons in the basal region of the adult SCO (paper III) raised the question of whether they might have any functional association with the SCO. The question of species differences was also taken into consideration.

By light microscopy a group of nerve cells ("ganglion pericommissurale") had previously been observed in a superolateral position to the SCO of the dog (Yamada et al., 1957) and the monkey (Hosaka et al., 1957). The relationship between these neurons and the group of SCO-associated neurons is unclear (paper IV). The presence of a small mesodiencephalic nerve cell cluster located dorsal to the rostral area of the human fetal SCO, as well as a miniature nerve connecting this nerve cell group and the pineal gland had been reported (Møllgård and Møller, 1973) and the existence of a similar

nerve was later established in sheep and rabbit fetuses as well (Møller et al., 1975). Apart from paper IV no electron microscopic studies have dealt specifically with SCO-associated neurons and nerve fibers in mammals, but certain comments can be found in various investigations on the SCO. Thus Stanka et al. (1964) found a single nerve cell in the hypendyma of the adult rat, and Isomäki et al. (1965) noted the presence of some nerve cells in the hypendyma of the calf.

1. SCO-associated neurons (paper IV).

The term "SCO-associated neurons" was introduced as a purely morphological term describing the nerve cells which nestle down very close to the hypendyma. These neurons were investigated in rabbit fetuses in various developmental stages and in neonatal rabbits. The SCO-associated neurons, first observed on day 17 of gestation, develop into a rostral mesodiencephalic nerve cell group situated in the area dorsal to the rostral-most part of the SCO and into a more caudal layer of single neurons which extends throughout the length of the SCO. A relationship of the mesodiencephalic neurons to the single layer of neurons located more caudally, to the pineal nerve dorsally, and to the neurons located in the floor of the pineal recess rostrally is suggestive from the pattern of development.

The nature and the input-output relations of mammalian SCO-associated neurons in various stages of development are unknown at present, but a series of fluorescence histochemical investigations on the SCO of fetal and neonatal rabbits revealed that the neurons are not monoamine-containing (Møllgård et al., unpublished observations). Acetylcholinesterase reactions should also be performed on the SCO region of fetal and neonatal rabbits in order to establish whether the SCO-associated neurons are related - phylogenetically or ontogenetically - to the group of acetylcholinesterase-containing nerve cells in the pineal

complex and subcommissural area of frogs (Wake et al., 1974) or to some , at present, unknown system. The group of SCO-associated neurons probably includes more than one neuronal type. In adult rabbits some of the smaller neurons in the group seem to be Gomori-positive. Thus a Gomori-positive reaction in the basal SCO region is not necessarily reflecting the presence of secretory material in SCO hypendymal cells.

Ultrastructural observations in rats and rabbits indicate that none of the SCO-associated neurons innervate the SCO-ependymal or hypendymal cells under normal conditions (see paper VIII).

2. Terminals identified by fluorescence microscopy (paper V).

The possible innervation of the mammalian SCO has been subject of much dispute, which at least in part is the result of marked species differences. Fluorescence histochemically a rich serotonergic (5-HT) innervation of the basal part of the rat SCO has been described (Fuxe et al., 1968; Björklund et al., 1972; Wiklund, 1974; Bouchaud and Arluison, 1977; papers VI, VII), which presumably is identical to the plexus of zinc-iodide positive nerve fibers observed in the same region by Stanka (1964).

Only one study has focussed attention to the establishment of the 5-HT innervation of the SCO in rats during postnatal development (Wiklund, 1974), and in spite of the well-known species differences in several SCO-parameters (e.g. secretory activity) no comparative studies were available. Therefore, the following investigation on ontogenetic and comparative aspects of the 5-HT innervation was initiated (paper V).

The serotonergic innervation of the rat SCO was studied fluorescence histochemically in neonatal and adult rats, Mongolian gerbils, mice and rabbits. A dense serotonergic plexus was observed in relation to the basal part of the SCO in adult rats as reported earlier. The basal SCO of the gerbil also received a 5-HT input, but the innervation was more sparse

and the nerve fibers exhibited weaker fluorescence than in the rat. In marked contrasts to the observations in rat and gerbil no evidence was found of a 5-HT innervation of the mouse or the rabbit SCO. Neonatally none of the investigated species, including rat and gerbil, showed serotonergic innervation of the SCO. The fluorescence microscopy findings were correlated to the Gomori-positivity of the various neonatal and adult subcommissural organs (see section C).

3. Terminals identified by electronmicroscopy (papers VI, VII)

Although there have been numerous electron microscopic studies of the rat SCO the ultrastructural equivalent of the dense 5-HT innervation, as demonstrated by fluorescence microscopy has been established only very recently (Bouchaud and Arluison, 1977; papers VI, VII). Previous electron microscopic studies concerned with the innervation of the mammalian SCO have suggested inputs by cholinergic (Leonieni and Rechart, 1972) and catecholaminergic and peptidergic fibers (Miline et al., 1969; Miline, 1974), but these hypotheses were based on rather non-specific methods for ultrastructural identification of nerve terminals.

Specific ultrastructural identification of serotonergic nerve terminals has been possible by recent developments in radioautographic and immunocytochemical techniques. The radioautographic technique is based on the detection of exogenously administered tritiated serotonin, which is preferentially accumulated by and retained in serotonergic neurons (cf. Descarries, 1975). The immunocytochemical methods visualize with specific antibodies the enzyme tryptophan hydroxylase, which catalyses the first step of the biosynthesis of serotonin (Pickel et al., 1976). These two techniques have the advantage of demonstrating intact nerve terminals which the third technique - the method of identification by specific neurotoxic degeneration - does not offer. On the other hand they have the disadvantage of not allowing as high a resolution as conventional electron microscopy. The impaired resolution of ultrastructural details is especially evident when

using the immunocytochemical techniques, where deposits of electron dense reaction product cover labelled terminals and organelles. However, in areas of CNS where the serotonergic boutons constitute but a small fraction of the innervation, the radioautographic and immunocytochemical techniques are clearly more advantageous for identification than chemically induced degeneration.

The first conclusive ultrastructural evidence of the presence and nature of the innervation of the rat SCO has been provided very recently by radioautography (Bouchaud and Arluison, 1977) and by the use of serotonin neurotoxins (papers VI, VII). The latter study also includes the first high resolution electron microscopic investigation of the synapses in the rat SCO.

Small bundles of fine varicose unmyelinated axons were found to penetrate from the neuropil of the deep hypendyma into the basal part of the SCO, where they established numerous synapses with hypendymal cells and basal processes of ependymal cells. Single axons or bundles containing up to 4 axons passed apically between hypendymal cells and basal processes of ependymal cells to reach the nuclear level of the ependyma, where they terminated by forming abundant synapses located very close to the nuclei of the SCO ependymal cells. Despite the diversity of postsynaptic sites - hypendymal cells, basal processes and perikarya of ependymal cells - the large majority of the synapses seemed to belong to one single class, as reflected by common vesicle morphology, width of synaptic cleft and configuration of pre- and postsynaptic membrane specializations.

Many presynaptic elements (axon terminals and varicosities) were analysed in serial sections, which revealed that the boutons are spindle-shaped or spherical with an average diameter of $0.5 \mu\text{m}$ (range $0.3 - 0.9 \mu\text{m}$), while the intervaricose segments of the axons averaged $0.25 \mu\text{m}$ in diameter. Favourable longitudinal sections of axons demonstrated varicosities separated by approximately $1.5 \mu\text{m}$ axonal length. The boutons contained a characteristic population of round and elongated

(elliptical) clear vesicles, which often appeared to fill them almost completely. In up to 30% of the sections through the presynaptic elements one or more large dense core vesicles were visible. Clear vesicles or vacuoles of a size similar to the large dense core vesicles were occasionally observed. Mitochondria and irregular profiles of smooth endoplasmic reticulum were usually present in the presynaptic boutons, whereas coated vesicles were infrequent.

Typical "synaptic" membrane specializations were regularly found. The synaptic cleft was about 20 nm wide and contained an electron dense material. The postsynaptic elements consisted most frequently of the somata of SCO cells, i.e. ependymal as well as hypendymal, but basal processes of SCO ependymal cells and various processes of SCO hypendymal cells were also identified as sites of contact. Distinct cytoplasmic densities of variable thickness (up to 20 nm) adhered to the postsynaptic membrane, and gave the junctions the appearance of Gray's type I synapses. Furthermore, the examination of serial sections indicated that all boutons engage in synaptic junctions, and that all SCO ependymal cells are in synaptic contact with nerve endings. The serial sections also showed that individual axonal varicosities often engage in synaptic contacts with more than one SCO cell, and boutons in synaptic contact with at least 3 different SCO cells were sometimes found. The presynaptic elements in the SCO were characteristically devoid of astroglial covering.

A quantitative investigation showed that a typical varicosity in synaptic contact with an SCO cell contained a population of approximately 85% clear, elongated vesicles with diameters of 45×60 nm, 15% clear, round vesicles of 50 nm diameter, and 1-2% large dense core vesicles with a vesicle diameter of about 85 nm and a dense core diameter of 50 - 55 nm. The mean size of the postsynaptic membrane specialization was found to be 0.5 μ m. Experiments with specific neurotoxic drugs revealed that the nerve terminals in synaptic contact with

the SCO cells are identical to the fibers of the serotonergic plexus identified fluorescence histochemically (see the next section).

4. Effects of serotonin neurotoxins on the innervation of the rat SCO (papers VI, VII).

The specific neurotoxic actions of 5,6-DHT and 5,7-DHT on central indolaminergic systems have been well documented by fluorescence histochemical and biochemical investigations (cf. reviews by Björklund et al., 1974; Baumgarten et al., 1977). A few studies describing the ultrastructural features of degeneration induced by these neurotoxins have also appeared (Baumgarten et al., 1972; Baumgarten and Lachenmayer, 1972). Both drugs are dihydroxylated analogues of serotonin, and their neurotoxic specificity is thought to depend on this structural similarity, which leads to preferential accumulation in serotonergic neurons by the specific uptake mechanisms these cells have for their endogenous transmitter. Intracellularly, however, the drugs are supposed to exert a non-specific cytotoxic effect to which not only indolaminergic neurons are susceptible (Baumgarten et al., 1977).

The effects of 5,6-DHT and 5,7-DHT on the innervation of the rat SCO were investigated fluorescence histochemically after periods of one to three weeks, i.e. at a time when the lesioned terminals could be expected to have disappeared (Björklund et al., 1973 b; Nobin et al., 1973). These studies showed that both neurotoxic drugs caused a virtually total destruction of the serotonergic SCO innervation (papers VI, VII).

Specific degeneration induced by neurotoxic drugs can be used in ultrastructural identification of monoaminergic terminals (cf. Bloom et al., 1971; Lorez and Richards, 1973; Baumgarten and Lachenmayer, 1974), but for this purpose shorter survival times are preferred (cf. Baumgarten et al., 1972). In our experiments we chose survival periods of 1, 2 or 3 days after administration of 5,6-DHT or 5,7-DHT in doses shown fluorescence histochemically to produce an efficient denervation of the SCO.

It was revealed that a majority of the affected terminals followed an easily distinguishable electron dense process of degeneration and that the boutons during the initial stages of degeneration remained in contact with their post-synaptic target, which facilitated identification of the 5,6-DHT and 5,7-DHT sensitive serotonergic synapses. A detailed study of thousands of thin sections from several animals revealed that the large majority of the thin varicose unmyelinated axons, which established synaptic contact with SCO cells were undergoing degeneration. It was concluded that the SCO of the rat receives a dense plexus of serotonin-containing nerve fibers which form typical synaptic contacts with the specialized ependymal cells of the SCO, and that these fibers probably constitute the only direct nervous input to the organ.

C. Serotonergic regulation of secretory activity of the SCO
(papers V, VI, VII).

The SCO is a secretory organ. The serotonergic innervation of the SCO is, therefore, presumably involved in a regulation of this secretory activity. Furthermore, since the serotonergic innervation forms the major, if not only, nervous input to the SCO in the rat, its influence on the organ can be expected to be powerful. Bouchaud and Arluison (1977) speculated that the 5-HT innervation might exert a stimulatory influence on the activity of the organ, whereas our studies have indicated that the 5-HT input inhibits both protein and glycoprotein synthesis as well as the release of secretory material of the SCO (papers V, VI, VII).

1. Ontogenetic and comparative aspects (paper V).

Secretory activity including both synthesis and release of secretory material is difficult to evaluate without a number of different techniques, but in a combined karyometric, histochemical and ultrastructural investigation of the rat SCO we observed that the Gomori-reaction provided reliable information about the secretory status of a given SCO (paper VI).

In general three sorts of Gomori-positive material can be distinguished histochemically in SCO cells; a fine granular substance is found throughout the cells, worm-like masses may be present in the same location, and some strongly reacting large granules accumulate either in the basal processes close to the blood vessels or in the apical processes adjacent to the ventricular surface (see Fig. 1, p. 24).

A weak Gomori-positive reaction was found in both apical and basal processes of the SCO of adult rat and gerbil as opposed to the pronounced reactivity found in mouse and rabbit. The SCO of neonatal rat and gerbil exhibited a distinct apical reactivity similar to that found in the SCO of neonatal rabbits, whereas the organ definitely had a lower reactivity in neonatal mouse (for more complete data which also includes unpublished observations, see diagram on p. 25).

The findings of this study indicate an inverse relationship between serotonergic innervation and the secretory activity in the SCO in some animal species (rat and gerbil), which postnatally develop a serotonergic innervation of the organ. These observations are in line with the results of our experiments with chemically induced denervation of the rat SCO (papers VI, VII), which also indicate that the serotonergic innervation exerts a powerful inhibition of the secretory and synthetic activity of the SCO.

RABBIT

RAT

NEWBORN

ADULT



Fig. 1. This diagram demonstrates the three morphologically distinguishable Gomori-positive reaction products. They differ from each other in localization, size, shape and staining intensity. The fine granular substance and the worm-like masses are dark-purple, while the large coarse granules turn virtually black in a Bock-reaction.

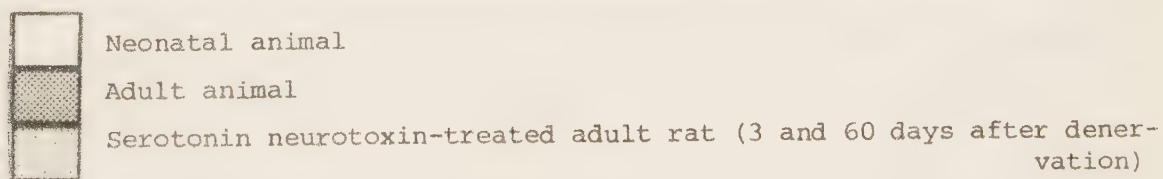
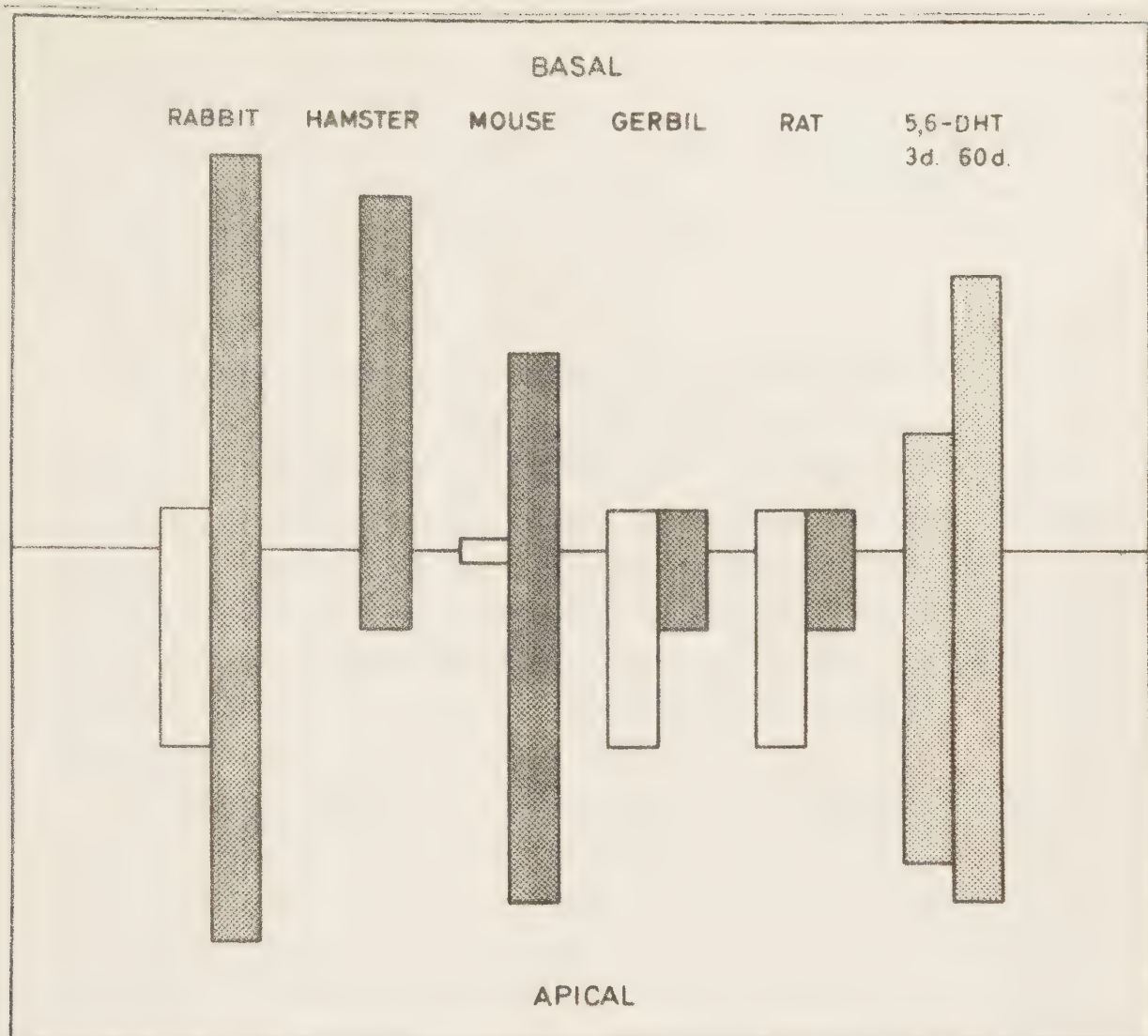


Fig. 2. Diagrammatic representation of the Gomori-positivity of the SCO of some neonatal and adult rodents and of some denervated rats. The upper half of the columns shows the intensity of the Gomori-reaction in the basal SCO, whereas the lower half shows the reactivity in the apical parts of the organ. A number of unpublished observations is included in the diagram. The decrease in stainability of the apical cytoplasm in the SCO of rats observed during the second post-natal week coincides with the establishment of the 5-HT innervation. Note that the rabbit SCO exhibits the strongest reactivity and that the SCO of a rat two months after denervation has a reaction pattern similar to that of the non-innervated mouse SCO. The SCO of the hamster shows a low apical

reactivity comparable to that of the rat and gerbil as opposed to a very strong basal reactivity. This interesting SCO-variation is under current investigation. All the material presented was stained together in the same sequence.

2. Denervation by serotonin neurotoxins (papers VI,VII)

The neurotoxic indoleamines could serve as tools for an analysis of serotonin neuron-target cell interaction, provided that the target cells are controlled mainly or exclusively by 5-HT synapses, the time sequence of the specific destruction is known, and the cellular activity of the target cells can be easily defined and measured. The SCO of the rat appears to fulfill the criteria for an ideal model of serotonin neuron-target cell interaction.

Secretory activity of the rat SCO: Although the SCO of the normal rat at the ultrastructural level shows apical secretory granules, flocculent material in the endoplasmic reticulum, and some scattered Golgi complexes, a comparison between the secretory activity of the rat and that of several other species indicates that the normal rat has a rather low secretory activity (see diagram, p.25). Thus, in a thorough electron microscopic study the complete absence of secretory elements in the basal processes was pointed out (Stanka et al., 1964).

The secretory product of the rat SCO is known to be PAS-positive, Gomori-positive and to contain protein (Olsson, 1958). These findings were confirmed (paper VI), and the results of the different histochemical reactions indicated the presence of glycoproteins. (Maltase-resistant PAS-positivity) combined with a neurophysin-like substance (the positive PFA-AB and Bock's reaction), which might well be the protein part of the glycoprotein, since the various reactions revealed parallel patterns of intensity and exhibited similar topographic distributions on serial sections.

Changes in secretory activity after denervation: Serotonergic synapses obviously exert a regulatory role in the rat SCO. As early as 24 hr after injection of 5,6-DHT or 5,7-DHT, we observed a tremendous engorgement of endoplasmic reticulum and an accumulation of pale and dense granules in apical, perinuclear, and basal parts of the organ. Histochemically, an increased reaction for neurophysin-like material and for glycoproteins in both apical and basal processes was evident. The reactivity was further enhanced four days after the neurotoxic treatment (and even more enhanced two months after treatment - see Fig. 2, p.25).

These phenomena might reflect either a stimulation of the synthetic machinery or an inhibition of release of secretory material or any combination of these possibilities. We found also, however, that the mean size (volume) of the SCO cell nucleus of 5,6-DHT-treated rats was significantly increased compared to that of control rats. This dramatic change in nuclear volume and the presence of large nucleoli, together with indications of an increased release of secretory material (many exocytotic and pinocytotic profiles and presence of flocculent material in the extracellular space), suggest that the synthetic machinery is stimulated under the experimental conditions. Whether the increased release of secretory material is secondary to increased production of this material or whether it is directly dependent on the lack of 5-HT input is not known at present but open to experimental verification.

Hypothetically, the cellular changes in the SCO could either be a denervation effect, or depend on some direct effect of the neurotoxic drugs on the SCO cells. These possibilities were investigated by injection of ^{14}C -labelled 5,6-DHT (paper VI). The ensuing radioautographic analysis revealed no accumulation into SCO cells, which presumably precludes the possibility of direct cytotoxic effects on the SCO cells. Furthermore, since no significant receptor effects have been reported for 5,6-DHT or 5,7-DHT, we concluded that the ob-

served cellular changes were responses to removal of the serotonergic innervation. This implies that the 5-HT input exerts a powerful tonic inhibitory control of the SCO, and that denervation removes this inhibition from the SCO cells, which respond with increased synthetic and secretory activity. Since the nerve terminals innervating the SCO contain large amounts of serotonin, the inhibition of the SCO activity is presumably mediated by release of this transmitter and its interaction with serotonergic receptors on the SCO cells. This conclusion needs, however, to be corroborated by more direct evidence, since it recently has been claimed that 5-HT neurons may contain putative peptide transmitters in addition to serotonin (Chan-Palay et al., 1978; Hökfelt et al., 1978).

The observed inverse relationship between secretory activity and serotonergic innervation is in line with previous observations from our ontogenetic and comparative studies which indicate that the 5-HT input to the SCO cells exerts a powerful inhibition on their protein synthetic machinery.

D. Non-monoaminergic reinnervation of the denervated SCO

(paper VIII).

It has been observed that after axotomy induced by specific neurotoxic drugs, central monoaminergic neurons have the capacity of regenerative growth, and are able to reestablish terminal innervation patterns (e.g. Nobin et al., 1973; Nygren and Olson, 1977; Wiklund et al., 1978). The regrowth of chemically lesioned axons is presumably favoured by a virtually unaltered tissue milieu, where postulated mechanisms of guidance and recognition presumably are intact. Several aspects of the regeneration of monoaminergic systems remain, however, to be clarified. For non-monoaminergic systems it has been well established by experiments in various CNS regions that destruction of one afferent system will often lead to plastic remodelling of the neuropil, which involves the production of new synapses by intact systems

to replace the synapses lost by the lesion. This phenomenon is generally known as reinnervation through collateral sprouting. Until now, it has not been known, however, if destruction of monoaminergic afferents to an area of CNS will elicit collateral sprouting in remaining afferents. In case this type of remodelling occurs, it will be of fundamental interest to determine if the regrowing monoaminergic nerve fibers in their reinnervation process replace the foreign terminals.

The denervated rat SCO served as an ideal model for such investigations (paper VIII). Neurotoxic destruction of the serotonergic SCO innervation by intraventricular administration of 5,6-DHT or 5,7-DHT was found to lead to an efficient reinnervation of the SCO cells by non-monoaminergic boutons. Even after very long survival times the yellow fluorescent fibers did not reappear. At the ultrastructural level, however, there was a gradual reappearance of synapses on the SCO cells. The reinnervating boutons were considerably larger, had a different synaptic vesicle content, and more mitochondria than the 5-HT boutons they replaced, but the synaptic junctions formed with the denervated SCO cells had the same ultrastructural appearance as the original 5-HT synapses. The new terminals did not degenerate after a second treatment with serotonin neurotoxins.

Apparently, the reinnervating boutons did not substitute the serotonergic innervation functionally, since the marked elevation of secretory activity caused by interruption of the inhibitory 5-HT mediated control (papers VI, VII) remained in spite of the total reinnervation observed. Presumably, the inhibition of the secretory activity of the SCO is mediated by interaction with specific receptors sensitive to serotonin.

The origin of the fibers replacing the lost 5-HT input has not yet been determined, but the boutons are very similar to afferents of the nearby SCO-associated neurons. It is possible that the observed reinnervation by foreign synapses explains why the regrowing serotonergic neurons fail to reestablish their connections with the SCO.

THE RAT SCO AS A MODEL IN NEUROBIOLOGY

Serotonin and its derivatives are supposed to modulate barrier properties. Therefore, it was tested whether differences in the 5-HT innervation of the SCO could be correlated with differences in the tight junction morphology of the organ. The findings in papers I and II together with information in paper V indicate that it is not the case, since both the neonatal and adult rabbit SCO (non-innervated) show the same tight junction morphology as the heavily innervated rat SCO. Therefore, it is unlikely that the observed changes in the tight junction network of the gerbil SCO during the development reflect the establishment of the 5-HT innervation.

The serotonergic innervation of the SCO of the adult rat where the serotonergic boutons form the major, if not the only, innervation of a target cells represents an extraordinarily simple system in the mammalian CNS. In other areas of the brain the serotonergic terminals represent only a small portion of the innervation of a given region, which means that functional exploration of the serotonergic input is complicated by the interaction of other systems. Our denervation experiments (papers VI, VII) show that the potent serotonergic regulation of SCO synthetic and secretory activity is readily accessible to histochemical, ultrastructural and karyometric methods. Taken together this indicates that the SCO innervation represents a unique model in pharmacological and physiological investigations of serotonergic innervation. It can be argued that the control of serotonergic activities in the SCO is not representative of the serotonergic systems, which innervate electrically active neurons. However, the observed serotonergic influence on tyrosine hydroxylase activity in the noradrenergic neurons of the locus coeruleus (Lewis et al., 1976; Renaud et al., 1975) may indicate that regulation of biosynthetic activities could be a common property of serotonergic innervation. We therefore believe that further exploration of the serotonergic

innervation of the rat SCO will yield important contributions to our understanding of serotonergic systems in general.

Specific neurotoxic destruction of the serotonergic innervation of the subcommissural organ of the rat is followed by an efficient reinnervation by collateral sprouting of non-monoaminergic axons, which normally do not innervate the SCO cells. Morphologically, the reinnervating fibers totally replace the serotonergic synapses lost by the lesion, but, functionally, they fail to substitute for the potent inhibitory control of secretory activity normally exerted by the serotonergic innervation. Thus, the rat SCO seems to be a highly suitable model for studying reinnervation by collateral sprouting.

Finally, the discovery of the serotonergic innervation and the suggested powerful inhibitory regulation of the secretory activity of the SCO opens the possibility of modulating the activity of the organ. Thus, pharmacological manipulations of the secretory activity with serotonergic agonists and antagonists could prove a very valuable tool in the future exploration of SCO physiology.

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